Reconstitution of stellacyanin as a case of direct Cu(I) transfer between yeast copper thionein and 'blue' copper apoprotein

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It was of interest to examine whether yeast Cu-thionein could be used to transfer the thiolate bound copper directly into the copper binding site of 'blue' apoproteins which contain free thiol groups. In particular apo-stellacyanin was used in the present study and it was found to be able to accept Cu(I) from yeast Cu-thionein, without any detectable unspecific Cu(II) intermediate, both aerobically and anaerobically.

Metallothionein *Yeast Cu-thionein* *Stellacyanin* *Cu(I)-transfer* *Copper*

1. INTRODUCTION

Copper thionein belongs to a group of uniquitous metal-thiolate rich proteins called metallothioneins. In the vertebrate metallothioneins (M_r 6500) Cu, Cd, Zn and to a lesser extent Hg are simultaneously bound [1–3]. Homogeneous Cu-thionein, shown to be similar to a fragment of the mixed vertebrate metallothionein [4] was found in *Neurospora crassa* (M_r 2200). From baker's yeast a Cu-thionein containing 4 copper atoms and 8 cysteinyl residues, of M_r 4800, was isolated [5,6]. The primary structure of the latter species [7] was different from that of the vertebrate metallothioneins. Nevertheless this Cu-protein is continued to be named Cu-thionein on account of the striking structural similarities of the copper binding centres with those found for vertebrate Cu-thioneins [8].

In all known thiolate rich proteins the metals are tetrahydrally surrounded by 4 sulphur atoms [9,10]. Although considerable progress was achieved in the structure elucidation, the functional side remains obscure. The involvement of the Cu-thiolate core in redox reactions in a way similar to that of the rubredoxins could not be convincingly demonstrated [11,12]. The currently discussed biochemical role is assigned to the control of metal transport [13]. Apart from the possible scavenging of toxic metals like Cd or Hg the molecular basis of Cu transport deserves special attention. The high binding capacity to proteins, its ability to create reactive oxygen species and its essential biochemical role in many oxidases demand for a subtle regulation of the cellular copper concentration. An attempt has been made to use *Neurospora crassa* Cu-thionein as a copper transferring protein for the type 3 Cu in tyrosinase and hemocyanin [14]. A direct transfer could not be observed, some pretreatment of the employed Cu-thionein was essential.

2. MATERIALS AND METHODS

S-Cereviseae was grown anaerobically in the presence of 1.0 mM $CuSO_4$ for 48 h at 25°C. The isolation of Cu-thionein was performed as earlier described [6] using sequential chromatography on Biogel P6, QAE Sephadex A-25, Sephadex G-75, again QAE Sephadex A-25 and desalting on Biogel P-10.

Stellacyanin was purified, following the procedure described by Reinhammar [15], from the acetone powder of the latex of the Japanese lacquer

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tree *Rhus vernicifera* (supplied by Saito & Co., Osaka, Japan). The apoprotein was obtained as earlier described [16] by dialysis against 20 mM KCN and 20 mM Na_2CO_3, pH 11. It contained less than 5% residual copper and could be reconstituted with $CuSO_4$ up to 85% of the native Cu content.

Copper was assayed on a Perkin Elmer atomic spectrometer (model 400 S) furnished with a HGA-76 B unit. Low temperature EPR spectra at X-band frequency were run on an E-9 Varian spectrometer equipped with a variable temperature control unit. Electronic absorption spectra were recorded on a Perkin Elmer spectrophotometer model 330.

Anaerobiosis was obtained by repeated cycles of evacuation-flushing with purified argon of the samples in a Thunberg apparatus sealed to an EPR tube and/or to a quartz spectrophotometric cuvette.

3. RESULTS AND DISCUSSION

Apostellacyanin was aerobically incubated 24 h with Cu-thionein (1/4 mol thionein/mol stellacyanin). A time dependent incorporation of cupric copper was detected both by electronic absorption and EPR spectrometry, up to an identical level as observed with copper sulphate (see methods). No extraneous copper bound to the protein was deduced from the EPR properties (fig.1). This is an important observation which would strongly support the conclusion of a direct transfer of thionein Cu(I) into the vacant copper binding site of stellacyanin. The oxidation to cupric copper must have occurred after the Cu(I) transfer, since no extraneous Cu(II) EPR signal was detected when thionein was in excess of the amount required to reconstitute stellacyanin. In comparing the redox potentials of either protein, stellacyanin is oxidized at +184 mV [17] while Cu-thionein requires +350 to +410 mV, depending on the used oxidizing agent, i.e., hexachloroiridate (IV) or hexacyanoferrate (III) [18], respectively.

In order to reject the possibility of an uncontrolled oxidation of Cu-thionein, the copper transfer was also performed anaerobically (fig.2). After 24 h a molar equivalent hexacyanoferrate (III) to apostellacyanin was added to the colourless solution, which resulted in the immediate appearance of

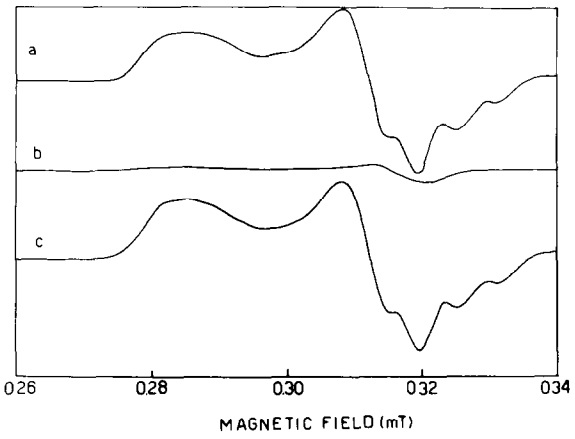

Fig.1. EPR spectra of stellacyanin. (*a*) 0.28 mM native enzyme; (*b*) 0.28 mM apoenzyme; (*c*) aerobically incubated with 0.15 mM Cu(I)-thionein in 20 mM borate buffer (pH 8.1). Instrument conditions: microwave frequency 9.15 GHz, microwave power 20 mW, temperature 77°K.

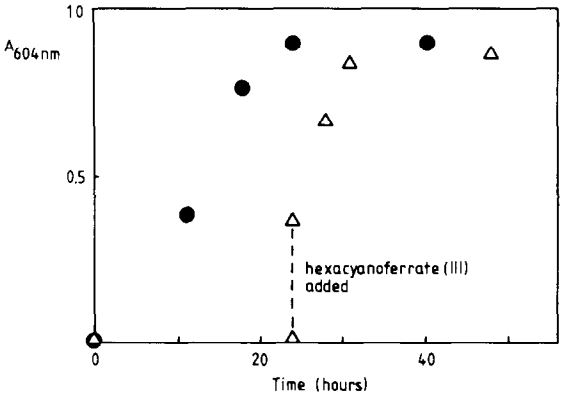

Fig.2. Reconstitution of apostellacyanin with Cu(I)-thionein. 0.28 mM apostellacyanin and 0.15 mM Cu(I)-thionein in 20 mM borate buffer (pH 8.1). (●) Aerobic incubation; (△) anaerobic incubation then 0.28 mM hexacyanoferrate added.

a portion of the expected blue absorbance, the remaining portion being recovered in a slower phase (fig.2). Apparently an equilibrium was established between Cu(I)-thionein and reduced stellacyanin, which was shifted by hexacyanoferrate (III) due to oxidation of stellacyanin.

It may be concluded that unlike the type 3 Cu binding centre of tyrosinase [14] the type 1 Cu binding site of stellacyanin binds cuprous copper much more strongly. In the former case the Cu-

thiolate-centre of Cu-thionein had to be irreversibly destroyed by oxidation prior to the incubation. The reported EPR spectrum was similar to that of yeast Cu-thionein [18] after the earlier described hexacyanoferrate (III) induced oxidation. The oxidatively destroyed Cu(II)-thionein had to be reduced again before incubation with tyrosinase. The thiolate groups remained oxidized, probably at the level of RSSR or even at higher oxidation states [8,18]. Following this pretreatment the less stable extraneously bound Cu(I) could then be transferred to apotyrosinase.

Altogether, this procedure appears to be poorly indicative of a specific copper transport role for the thiolate-Cu(I) centres of thioneins. In contrast the experiment with stellacyanin unequivocally indicated that oxidation of thionein is not required for copper transfer to a vacant type 1 Cu site.

These results provide no evidence that Cu-thionein is a specific Cu-transport protein in vivo, as they were obtained with functionally unrelated isolated proteins. However, they show that the Cu-thiolate centres of thionein are capable of transferring Cu(I) as such to a vacant site of other Cu proteins. It is conceivable to speculate that some protein centres have absolute demand for Cu(I) during biosynthetic processes. While this is unlikely in the case of stellacyanin, which can be reconstituted with Cu(II) [16], different structural situations may occur with other proteins, that make Cu(I) more suitable for binding. As many low-molecular weight and non-macromolecular complexes of Cu(I) are not stable, it is interesting to find that thionein can keep Cu, and even transfer it, as Cu(I) in aqueous solution and in the presence of air.

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